EXHIBIT

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Targeted replacement of normal and mutant *CFTR* sequences in human airway epithelial cells using DNA fragments

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Recent studies have reported that mutant genomic cystic fibrosis (CF) transmembrane conductance regulator (*CFTR*) sequences can be corrected in transformed CF airway epithelial cell lines by targeted replacement with small fragments of DNA with wild-type sequence. To determine if the observed genotype modification following small fragment homologous replacement (SFHR) was limited to transformed CF cell lines, further studies were carried out in both transformed and non-transformed primary normal airway epithelial cells. The endogenous genotype of these normal cell lines was modified following liposome or dendrimer transfection using DNA fragments with ΔF508 *CFTR* sequence (488 nt, complementary single strands) designed to also contain a unique restriction enzyme cleavage site (*XhoI*). Replacement at the appropriate genomic locus by exogenous ΔF508 *CFTR* DNA and its expression as mRNA was demonstrated by PCR amplification of genomic DNA and mRNA-derived cDNA as well as *XhoI* digestion of the PCR products. These studies show that SFHR occurs in both transformed and non-transformed primary human airway epithelial cells and indicate that single base substitution (the silent mutation giving rise to the *XhoI* site) and deletion or insertion of at least three consecutive bases can be achieved in both normal and CF epithelial cells. Furthermore, these studies reiterate the potential of SFHR as a strategy for a number of gene targeting applications, such as site-specific mutagenesis, development of transgenic animals, development of isogenic cell lines and for gene therapy.

INTRODUCTION

Targeting of genomic sequences by replacement with homologous DNA segments can be used to modify specific genes within chromosomal DNA (1–6). As such, it is possible to introduce portions of genes that permanently alter specific endogenous gene sequences via gene disruption and/or introduce specific mutations. Results from these types of experiment have facilitated assessment of gene structure and function in addition to the creation of animal models of disease (4,7–12). Alternatively, restoration of gene function through targeted correction of specific mutations has significant potential as an approach to gene therapy (1,13–15).

While the mechanism(s) underlying homologous replacement in mammalian systems is not clearly understood, the low frequency of successful targeting (10^{-6}) suggests that there may be factors inherent in classical protocols that interfere with replacement. One factor may be the degree of non-homologous sequence associated with the targeting DNA segment. Classical approaches to gene targeting employ vectors that contain the genomic homolog of the targeted region as well as selectable marker genes (e.g. neomycin resistance, herpes simplex virus, thymidine kinase, hypoxanthine-guanine phosphoribosyl transferase) to select cells that have undergone homologous replacement (4,5,16–18). In addition to these selection marker genes, other non-homologous sequences that are part of the vector are also directly associated with the targeting DNA segment. The introduction of such sequences into cells in conjunction with genomic homologs could thermodynamically undermine the potential for homologous pairing and thereby decrease the probability for homologous exchange. Clearly, it would be preferable to decrease the degree of non-homology. Ideally, an approach that used only critical or 'essential' sequences should enhance the potential for an exchange at the appropriate locus (16).

One recent study directed at targeting the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene used relatively small fragments (–500 nt) of complementary, single-stranded (ss)DNA to correct the most common mutation in the *CFTR* gene, a 3 bp deletion in exon 10 (ΔF508) (13). These studies were carried out in transformed CF airway epithelial cells and showed that such a gene targeting technique had promise for site-directed alteration of genomic DNA (19). This small fragment homologous replacement (SFHR) strategy successfully
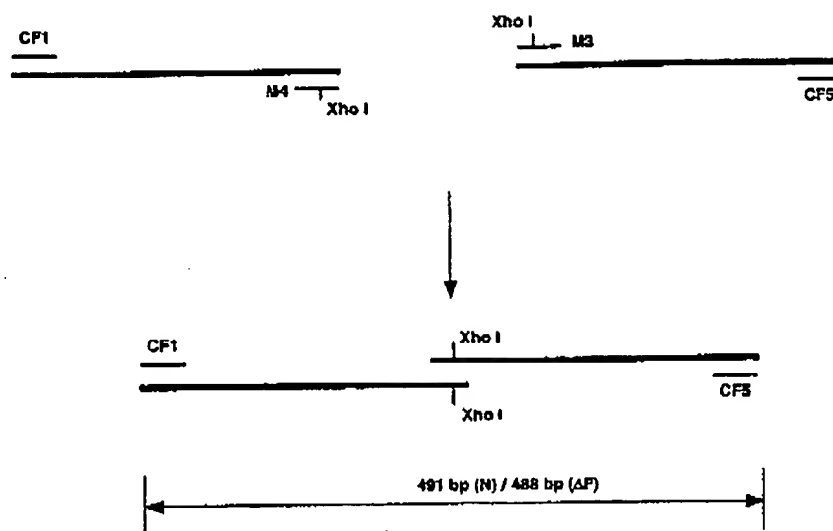
Figure 1. Diagrammatic representation of the generation of 491 and 488 bp fragments that contain wtCFTR and ΔF508 CFTR sequences, respectively. Fragments also contain a second mutation in exon 10 (a G→C conversion at bp 197 of the 491 bp fragment) that gives rise to a XhoI site. This mutation is in the third base of codon 11 (in exon 10) and does not change the amino acid determined by this codon. The M3 primer is a 21 base sense (+) oligonucleotide (5′-GATTATGGGAGAACTC-GAGCC-3′) that has been generated with the G→C conversion at base 197 and comprises bases 182–202 of the sense strand of the 491 bp fragment. The M4 primer is a 21 base antisense (−) oligonucleotide (5′-ACCCTCTGAAGGCTCGAGTTC-3′) that has a C→G conversion at base 197 of the antisense strand and comprises bases 212–192 of the antisense strand of the 491 bp fragment. The initial amplification with CF1/M4 and M3/CF5 gives fragments that were 212 and 310 b.. respectively, for the wtCFTR sequences. For ΔF508 CFTR sequences, the initial amplification gives fragments CF1/M4 (212 bp) and M3/CF5 (307 bp), because the ΔF508 mutation deletes bases 293–295 of the 491 bp wtCFTR fragment. Restriction digestion of the 491 or 488 bp fragments with XhoI yields digestion fragments of 199 and 292 or 289 bp, respectively.

corrected mutant sequences in ~1 in 100 cells, a range that is potentially therapeutic (20,21).

The studies presented here extend our previous investigations and evaluate the feasibility of using SFHR in normal human epithelial cells to modify four bases in genomic DNA. SFHR was analyzed in non-transformed and transformed normal airway epithelial cells with DNA fragments (488 nt) designed to target the exon 10 region of CFTR and delete three consecutive bases as well as effect a base conversion within the exon. In addition, SFHR studies were performed on transformed CF cells in order to evaluate whether the insertion of three consecutive bases would be influenced by a base conversion −100 nt upstream.

Allele-specific PCR amplification of genomic DNA and mRNA-derived cDNA demonstrated both replacement by exogenous CFTR DNA at the appropriate genomic locus and its subsequent expression as mRNA. Restriction enzyme digestion at the site of the silent mutation produced by the base exchange provided additional evidence that the gene targeting was successful. Control reconstitution experiments were also performed to substantiate the integrity of the PCR amplification assay (22–24). These studies indicated that no template switching had occurred and that the detected homologous exchange was not an artifact.

RESULTS

Transformed ΔF508 cells: DNA analysis

To determine if the non-homology introduced by the silent mutation resulting in the XhoI restriction site eliminated the ability of SFHR to insert three bases into genomic DNA, studies

were initially carried out in two transformed CF cell lines, CFPAC-1 and ΣCFNPE14o- (25,26). Targeted replacement was detected by PCR amplification using specific primers (Table 1) in both cell lines after transfection with a newly engineered 491 nt wtCFTR fragment, modified from previous experiments to contain a XhoI site (Fig. 1). The fragment was transfected as a Starburst dendrimer–DNA complex. Genomic DNA, isolated from transfected cells, was subjected to an initial round of PCR amplification with primers (CF1B/CF6; Table 1) that were not allele-specific and were located outside the region of homology defined by the 491 nt fragment. A secondary, allele-specific, amplification was carried out with primers CF1 and CF7C using the primary amplification product as template. Primer CF7C is specific for the normal allele. The PCR product from the secondary amplification was then digested with XhoI. Restriction digest analysis showed cutting in all the transfected samples (Fig. 2) and indicates that the additional non-homology within the 491 nt fragment, due to the presence of the unique restriction site, does not interfere with site-directed replacement.

Transformed N cells: DNA analysis

Analysis of transformed normal human epithelial cells (16HBE14o-) transfected with the ΔF508 DNA fragment (488 nt) in a cationic liposome (Lipofectamine) complex showed successful modification of endogenous sequences by the exogenous sequences. Genomic DNA from transfected cells was isolated and subjected to the two-step nested PCR analysis described above. Secondary amplification with primer pair CF1/CF8C, where one primer (CF8C) is specific for the ΔF508 allele,
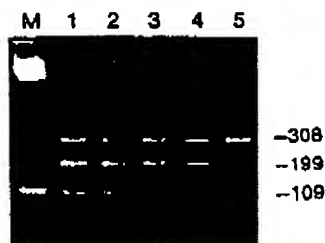
Figure 2. PCR and restriction enzyme analysis of transformed CF epithelial cells (ΣCFNPE14o- and CFPAC-1) transfected with 491 nt wtCFTR fragments containing the XhoI restriction enzyme site. CFPAC-1 (lanes 1 and 2) and ΣCFNPE14o- (lanes 3 and 4) cells were transfected with DNA–dendrimer complexes. Fragments were uncoated (lanes 1 and 3) or RecA-coated (lanes 2 and 4). Genomic DNA was first amplified with CF1B/CF6, producing fragments ~770 bp in length. These fragments were then isolated and amplified with CF1/CF7C to yield a 308 bp wild-type product (lanes 1–4). XhoI digestion gave rise to the 199 and 109 bp fragments (lanes 1–4). The PCR product, using primers CF1/CF7C, of non-CF (normal) control DNA was of the correct size (308 bp) but was not cut by XhoI (lane 5). Incomplete cutting of the PCR product could be the result of the presence of PCR amplification components that inhibit the activity of XhoI (45).

followed by XhoI digestion showed that exogenous sequences were present at the appropriate *CFTR* locus within the population of transfected cells (Fig. 3). Exogenous sequences were detected in genomic DNA isolated from cells subcultured three times after transfection when analyzed by PCR. Given that these cells average about seven doublings (generations) per subculture (27), the homologous exchange was shown to be present for at least 21 generations following transfection.

Table 1. PCR primers for SFHR analysis

Primer	Sequence	Location
CF1 (S)	5'-GCAGAGTACCTGAAACAGGA-3'	Intron 9
CF1B (S)	5'-CCTTCTCTGTGAACCTCTATCA-3'	Intron 9
CF5 (A)	5'-CATTCACAGTAGCTTACCCA-3'	Intron 10
CF6 (A)	5'-CCACATATCACTATATGCATGC-3'	Intron 10
CF7B (S)	5'CCATTAAAGAAAATATCATCTTTGG-3'	Exon 10
CF8B (S)	5'-CCATTAAAGAAAATATCATTGG-3'	Exon 10
CF7C (A)	5'-ATAGGAAACACCAAAGATGA-3'	Exon 10
CF8C (A)	5'-ATAGGAAACACCAATGATAT-3'	Exon 10
CF17 (S)	5'-GAGGGATTTGGGGAATTATTTG-3'	Exon 9
CF22 (A)	5'-CTTGCTAAAGAAATTCTTGCTC-3'	Exon 9

The nucleotide sequences of the *CFTR* DNA primers are described in the text and were derived from published data (42,44,46). Sense (S) and antisense (A) primers are as indicated.

Non-transformed primary normal cells: DNA analysis

Direct, allele-specific PCR analysis (ΔF508-specific) of genomic DNA from non-transformed primary normal airway epithelial cells (HTE11) transfected with 488 nt ΔF508 DNA fragments showed that the PCR products were cleaved by XhoI and that homologous replacement occurred regardless of whether the fragments were uncoated or coated with RecA (Fig. 4). Cells were
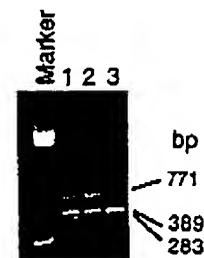

Figure 3. PCR and restriction enzyme analysis of transformed normal epithelial cells (16HBE14o-) transfected with the 488 nt (ΔF508) ssDNA fragments using Lipofectamine (1:8 negative to positive charge ratio or 28.8 μl Lipofectamine to 4 μg DNA). PCR was first performed on isolated DNA using non-allele-specific primers (CF1B/CF6), producing a 771 bp fragment for wtDNA and a 768 bp fragment for ΔF508 DNA. This product was then isolated using spin columns (Qiagen, Chatsworth, CA) and allele-specific PCR was performed (CF1B/CF8C), which produces a 389 bp fragment. This fragment was then subjected to XhoI restriction enzyme digestion, producing 283 and 106 bp fragments. The 771/768, 389 and 283 bp products can be detected on the gel. The marker lane is a 123 bp ladder (Gibco BRL). Lane 1, DNA from the first passage post-transfection: lane 2, the second passage; lane 3, untransfected 16HBE14o- cells (amplified with primers CF1B/CF7C). This fragment does not cut with XhoI.
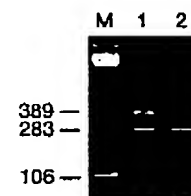

Figure 4. Amplification of DNA from HTE11 cells transfected with uncoated (lane 1) or RecA-coated (lane 2) ΔF508 (488 nt) fragments complexed with dendrimer. The CF1B/CF8C primers gave rise to a 389 bp (ΔF508) product (lanes 1 and 2). Digestion with XhoI produced fragments of 283 and 106 bp.

transfected with fragments as a Starburst dendrimer–DNA complex and PCR amplification was performed with primers CF1B/CF7C. The resultant PCR product was subsequently digested with the restriction enzyme XhoI.

Non-transformed primary normal cells: RNA analysis

Expression of CFTR mRNA containing the ΔF508 mutation was also detected in HTE11 cells transfected with the 488 nt fragment following allele-specific RT–PCR amplification of total RNA (Fig. 5). PCR amplification of mRNA-derived cDNA from controls (non-transfected HTE11 cells) with either the wild-type-(CF17/CF7C) or ΔF508-specific (CF17/CF8C) primers indicated a PCR product only after amplification with wild-type-specific primers (CF17/CF7C). On the other hand, PCR products were present after RT–PCR amplification of mRNA from transfected HTE11 cells with both wild-type- and ΔF508-specific primers, indicating a mixed population (normal and ΔF508) of CFTR mRNA. In addition, only products from the CF17/CF8C amplification were cleaved by XhoI. Because the primers used amplify across intron–exon boundaries, the results cannot be attributed to
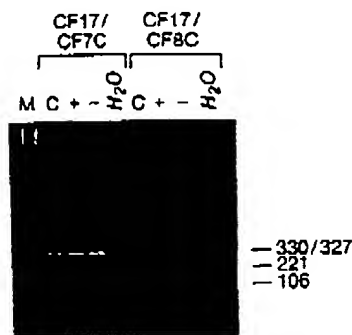
Figure 5. RT–PCR analysis of CFTR mRNA from HTE11 cells transfected with uncoated (–) or RecA-coated (+) 488 nt fragments as a dendrimer–DNA complex. The cDNA was amplified with CF17 (exon 9) and primers for normal (CF7C, 330 bp fragment) or mutant (CF8C, 327 bp fragment) sequences. Digestion with *Xho*I only showed the expected 221 and 106 bp restriction fragments for DNA amplified with CF17/CF8C. Non-transfected controls are in lanes C. Marker DNA (123 bp) is in lane M. No PCR products were detected in the absence of RT (data not shown).

Figure 6. Reconstitution experiment with DNA from non-transfected CFPAC-1 cells mixed with 491 nt wt*CFTR* fragment. Fragments were mixed with cellular DNA at concentrations that are equivalent to different ratios of fragments (10^6–10^{-1}) per cell. PCR analysis used the allele-specific primers CF7B/CF6 to detect the presence of normal sequence. Control PCR was carried out using the appropriate allele-specific primers for both CFPAC (CF8B/CF6) and 16HBE14o- (CF7B/CF6) DNA. There is no evidence of any PCR product in any of the reconstitution samples.

amplification of genomic DNA. These analyses confirm that genomic DNA sequences, replaced at the exon 10 locus of *CFTR* with exogenous sequences, will be expressed as mRNA in non-transformed primary human airway epithelial cells.

Reconstitution studies

PCR amplification of genomic DNA isolated from non-transfected CF cells (ΔF508/ΔF508) incubated with different amounts of 491 nt wt*CFTR* fragment resulted in the absence of detectable PCR product under identical conditions used to verify homologous replacement (Fig. 6). The number of fragments in these mixtures ranged from the equivalent of 1 000 000 copies/cell (assuming 5 pg DNA/cell) to 0.1 copies/cell. Non-specific amplification of wt*CFTR* did not occur regardless of whether fragments were coated with RecA or when wt*CFTR*-specific primer pairs, e.g. CF17/CF7C, were used (data not shown).

DISCUSSION

This study reiterates our previous findings (13,19) and indicates that specific genomic DNA sequences can be modified by SFHR. Specifically, these studies demonstrate homologous replacement of the exon 10 locus of *CFTR* in transformed and normal CF as well as non-transformed primary normal human airway epithelial cells. These findings, and earlier studies that show functional correction of CF epithelial cells by small fragments of DNA (13,19), indicate that SFHR has potential as an approach for gene therapy of CF and as a general sequence-specific gene modification strategy in isolated cells. In particular, SFHR could be used to develop isogenic cell lines, clonal populations of cells derived from a single parental line, with either a CF or normal genotype and then modified at the *CFTR* locus by SFHR to contain specific genomic mutations on the same genetic background.

Previous SFHR studies indicated a high efficiency of replacement, with ~1 in 100 transformed CF airway epithelial cells showing site-specific modification by the insertion of three bases that corrected the ion transport defect associated with CF (13).
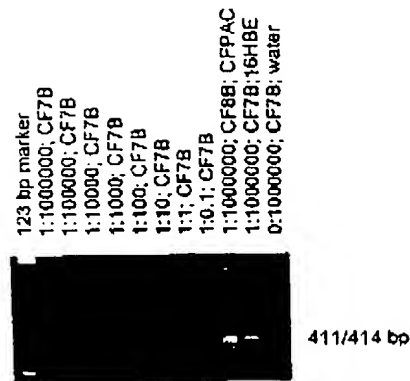
This value is significantly higher than that achieved with classical approaches to gene targeting (5,16,28). Other studies investigating gene targeting have taken several different approaches. One study, which used oligonucleotide ssDNA (40 nt), reported a replacement frequency of 10^{-5} in episomal vector sequences (3). However, it is only a strategy that has used DNA–RNA chimeric oligonucleotides to specifically alter alkaline phosphatase, β-globin, H-*ras*, K-*ras* and factor IX sequences by base conversion (29–32) and an early study in embryonic stem cells (34) that indicate gene targeting frequencies similar to those observed by SFHR.

The higher frequency observed with SFHR could, in part, be due to a number of factors, including fragment length, the degree of homology or the single-stranded nature of the fragment. Logic dictates that the thermodynamic potential for homologous pairing is enhanced when the degree of homology associated with the exogenous or therapeutic fragment approaches 100%. Since the targeting fragments used in SFHR are almost 100% homologous to the region being targeted, the residence time for the fragment at the target locus should be increased and, therefore, should increase the probability for the appropriate recombinatory enzymes to effect homologous exchange. The parameters influencing this homologous pairing will need to be evaluated empirically to determine the extent to which they will modulate the frequency of homologous exchange and are the subject of future studies.

The reported frequency of SFHR reinforces the therapeutic potential of this strategy, especially, as is the case in CF, when restoration of function in a portion (5–10%) of the cells is sufficient to correct a mutant phenotype within an epithelial monolayer (20,21). It was not possible to determine the frequency of targeting events in transformed or non-transformed primary normal cells functionally, as a single targeting event gives a heterozygous genotype that is phenotypically indistinguishable from that of wild-type cells. Based on data from our previous results using SFHR (13), a homozygote for the ΔF508 mutation

would theoretically occur at a frequency of ~1 in 10 000 cells and it would therefore be difficult to accurately determine the frequency.

There are a number of considerations to optimizing this technique. First, assessment of successful SFHR can be undermined when the genotypic modification does not result in measurable phenotypic change. This has been a limiting factor in the use of *CFTR* as the target for SFHR. Although corrected cells show cAMP-regulated Cl efflux by patch clamp, these measurements are tedious and not readily amenable to analysis of large numbers of cells. The PCR analyses described within the text were developed to specifically detect successful homologous exchange. While there is a limited potential for the production of artifacts, depending on the PCR conditions, non-specific or aberrant amplification does not appear to occur using the allele-specific conditions described here (Fig. 6). No evidence of a false positive signal was detected even when genomic DNA was amplified in the presence of fragment at proportions up to 1 000 000 fragments/cell. This ratio represents a 100% efficiency of fragment delivery based on the reported conditions for transfection. From previous studies only 30–50% of the cells in a given population have been shown to be effectively transfected by Starburst dendrimer (34). Additional studies indicate that <5% of transfected fragments enter the nucleus (~50 000 fragments/nucleus) even under optimal conditions using Lipofectamine (35). In addition, PCR assessment of targeted replacement at the end of the 5 h incubation/transfection period did not detect homologous replacement (K.K. Goncz, unpublished data).

Another factor that will influence optimization is fragment preparation. Currently, PCR is used to generate the fragment and gives a relatively low yield (1–3 µg fragment/100 µl reaction). While there is a possibility that errors may be introduced into the fragment sequence during amplification, recent work has shown that PCR-generated fragments are adequate for transfection (36). In addition, proofreading polymerases, such as Pfu (Stratagene, La Jolla, CA) can also be used.

In summary, SFHR has potential as an approach to targeted replacement of gene sequences. Through SFHR it is possible to insert or delete up to three consecutive bases in genomic DNA. Moreover, both insertion and deletion can be linked to a base conversion, a single base G→C, ~100 nt upstream. This observation indicates that both events can occur simultaneously, even though they may involve different mechanistic pathways. These findings, coupled with our earlier studies showing functional correction of CF epithelial cells after SFHR (13), add further support to the development of this technique for gene therapy of inherited genetic diseases. The work presented here, in conjunction with other gene targeting studies (1,3,29, 31,32,37,38), indicates that alternative gene targeting strategies with DNA fragments can be used for the site-specific alteration of genomic DNA.

MATERIALS AND METHODS

Cells and culture conditions

SV40-transformed normal tracheobronchial (16HBE14o-) (39) and CF nasal polyp (ΣCFNPE14o-) (26) cell lines, as well as non-transformed primary normal airway (HTE11) epithelial cells (40), were used. In addition, an immortalized pancreatic adeno-

carcinoma cell line (CFPAC-1) (25) was used. All CF cells were homozygous for the ΔF508 *CFTR* mutation (ΔF508/ΔF508). The SV40-transformed cell lines were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. CFPAC-1 cells were grown in Dulbecco's modified Eagle's/Ham's F12 medium (1:1) (DME/F12). Non-transformed HTE11 epithelial cells were grown in modified LHC8e (MLHC8e) medium (40). All cells were grown under humidified conditions in a 5% CO_2 atmosphere at 37°C on cell culture dishes coated with fibronectin (FN)/Vitrogen (V)/bovine serum albumin (BSA) (40,41).

Fragment preparation

The fragment was prepared as previously described (13), except that the template used for amplification was engineered by PCR-mediated site-directed mutagenesis to contain a silent mutation (G→C at base 33 in exon 10) that gives rise to a *XhoI* restriction site (Fig. 1). The template was generated using overlapping oligonucleotide primers, M3 and M4, containing sequence corresponding to *CFTR* exon 10 with the appropriate base change to give the *XhoI* restriction enzyme cleavage site, used in conjunction with primers CF1 and CF5 (Table 1) (13). As a template, plasmid T6/20 (42–44) was used to generate the wild-type fragment (491 bp) and lymphocyte DNA from a ΔF508 homozygote was used for the ΔF508 fragment (488 bp). The final 491 and 488 bp fragments, containing the *XhoI* site, were generated by combining the isolated products from the CF1/M4 and M3/CF5 amplifications in a PCR extension reaction without primers for five cycles (0.5 µM primers, 50–100 ng DNA, 2.0 mM Mg^{2+}; 95°C for 60 s, denaturation; 58°C for 60 s, annealing; 72°C for 90 s, extension; Perkin Elmer Thermal Cycler 9600). Primers CF1 and CF5 were then added to generate full-length fragment in another PCR amplification under the same conditions as above, except that the PCR amplification was performed for 30 cycles with 5 min extension on the last cycle. The resulting fragments were ligated into pTA cloning vectors (InVitrogen, San Diego, CA), sequenced and used as a template to generate the fragment for transfection.

The fragment used in the transfections was prepared by PCR in a manner similar to that previously described (13). Template (pTA491 or pTA488) was added as a 10 ng aliquot per 100 µl PCR amplification. Fragment size was confirmed by 1% agarose gel electrophoresis. Both the 491 and 488 bp products were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 2.5 vols ethanol. DNA fragments were heat denatured. The resulting 491 or 488 nt complementary ssDNA was then used in the targeted replacement studies. The fragments were either left uncoated or coated with RecA protein (Pharmacia, Piscataway, NJ) by a protocol modified from that previously reported (13,19). Briefly, DNA fragments (20 µg) were denatured at 95°C for 10 min and then immediately placed in an ice–water bath. A 4 µl aliquot was then added to 37.3 µl reaction buffer containing 160 µg RecA protein, 5.4 mM ATPγS, 5.4 mM Mg acetate and 3.7 µl 10× reaction buffer (100 mM Tris–acetate, pH 7.5 at 37°C, 10 mM dithiothreitol, 500 mM Na acetate, 50% glycerol and water) and incubated for 10 min at 37°C. The reaction was terminated by addition of 7.7 µl 200 mM Mg acetate. After coating, the fragments were immediately placed on ice until transfection (10–60 min). Uncoated fragments were kept on ice in the presence of reaction buffer (without RecA).